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Application of spectrofluorometry for evaluation of dry powder inhalers *in vitro*

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Andersen cascade impactor (ACI) is commonly used for the testing of pharmaceutical aerosols, which has to be coupled with an instrument for quantitative analysis of drug depositing on each stage of the ACI. This procedure consumes much time in operation. Therefore, this study was aimed at speeding up the process of drug analysis in aerosol formulations after obtaining samples from the ACI. From the results obtained, it was proved that the validated spectrofluorometric method was accurate and sensitive. It was capable of giving similar results to those we obtained from HPLC-UV analysis. There was no interference from the amount of lactose carrier incorporated in the formulation in the step of salbutamol analysis indicating specificity of the method. As a result, samples were analyzed without further separation. The detection limit was 0.1 μg/ml. Hence, spectrofluorometry can be used as a substitute method to HPLC-UV in determining the small quantity of salbutamol after aerosolization from dry powder aerosols. The present study suggests that spectrofluometry can be a rapid and efficient method in the pharmaceutical analysis of aerosols.

1. Introduction

In general, evaluation of dry powder inhalers (DPI) employs either twin stage impinger (TSI) or Andersen Cascade Impactor (ACI). TSI is a sizing instrument that is based on liquid impingement whereas ACI relies on impaction. Both sizing instruments justify aerosols on their aerodynamic diameters [1–3]. Cascade impactor is the dominant technique used to assess aerodynamic parameters in routine quality control and research during the development of new aerosol products. One advantage of inertial impaction is that it measures aerodynamic diameter, the parameter thought to be most relevant to lung deposition – the ultimate goal of aerosol therapy. Cascade impactors are widely used for measuring the size distribution of airborne particles. The device separates aerosol samples into nine size intervals. The distribution of particle size impacting on a particular stage depends on a jet velocity of the stage and the cut-off diameter of the stage above it. Large particles with sufficient velocity and inertia tend to impact whereas small particles follow the air stream around the edge of the plate to the next stage, where they either impact or pass onto the next stage. Progressively smaller particles impacting on succeeding stages will be obtained. The drug particles are collected for those stages and analyzed by chromatographic and spectrophotometric means to determine their mass. Multistage impactors are popular because more information is recovered about the range of particle size within the distribution. Reconstruction of the distribution based on the calibration data allows for calculation of the mass median aerodynamic diameter (MMAD). These techniques have the advantage of sampling the entire aerosol and allowing chemical analysis of the drug. The drawback is that this requires considerable manual labor to perform the time consuming analysis.

Generally, dry powder formulations contain lactose as a carrier with drug particles interact. Thus, it is difficult to separate those two components. Therefore, a separation technique is necessary before an analysis such as liquid chromatography, i.e. HPLC-UV. Nevertheless, this technique is time consuming compared to other non-separating techniques such as UV or fluorescence analysis. However, UV is not sensitive enough to analyze a microgram concentration of salbutamol. Loper Paz and Townshend [4] have also reported that lactose and glucose interfered with the determination of imipramine and chlopromazine. Therefore, the potential interference of lactose on the determination of salbutamol has been investigated.

Salbutamol, a β_2 adrenoceptor agonist, is the most commonly used bronchodilator. Among others, the drug is formulated as aerosol as it is directly delivered to the target organ in low doses. Boulton and Fawcett [5] used fluorescent properties to analyze salbutamol *in vivo* from biological samples. To the best of our knowledge, no direct fluorescent determination of salbutamol has been previously reported in the literature. Most analytical methods for aerosol products are similar to those used for other dosage forms, including chromatographic techniques for determining the mass of drug or excipient. However, because of

the unique delivery characteristics, aerosol products require special collecting methods and apparatus for assessing the uniformity of dosage units and particle size distribution. The ingredients of the formulation can be recovered by washing the collection container. The samples are typically quantified by HPLC equipped with UV methods after the delivery of a single dose into the impactor. Although gravimetric method is an alternative, there is a limit to the presence of a single powder component and, at the same time, a high sensitivity balance is needed. Therefore, it requires chemical detection methods to measure drug deposition on each of the stage of the impactor. In practice, the concentration range for a standard curve and its correlation coefficient and linearity must be determined. The coefficient of variation of the assay, interday, intraday of coefficient of variation, the detection limit must be established for the method to be validated.

The aim of this work was to develop a sensitive spectrofluorometric method for the determination of salbutamol susceptible to interference from lactose carrier in aerosol analysis *in vitro*. We developed a spectrofluorometry method which is compared to conventional HPLC-UV to evaluate single dose delivery of DPI. Simple methodologies are described based upon spectrofluorometry for the evaluation of salbutamol in pharmaceutical aerosols. Two formulations of dry powder aerosols were employed with different amounts of lactose carrier. Two different flow rates were used to aerosolize aerosols.

2. Investigations, results and discussion

2.1. Dry powder formulation uniformity

Both dry powder formulations were uniform and homogenous with a 99.95% nominal dose of salbutamol analyzed by HPLC-UV. The dry powder formulation was viewed by SEM and investigated by X-ray microanalysis (Fig. 1). The micrographs showed that the drug particles adhered on the surface of the coarse lactose carrier. Salbutamol particles were uniformly dispersed around the carrier particles. One way to explain this is that adhesion between drug particle and carrier is enough to give a uniformity of distribution. It is expected that when the formulation is aerosolized, the drug particles will be delivered by the detachment forces from the carrier surface and hence travel to the target organ.

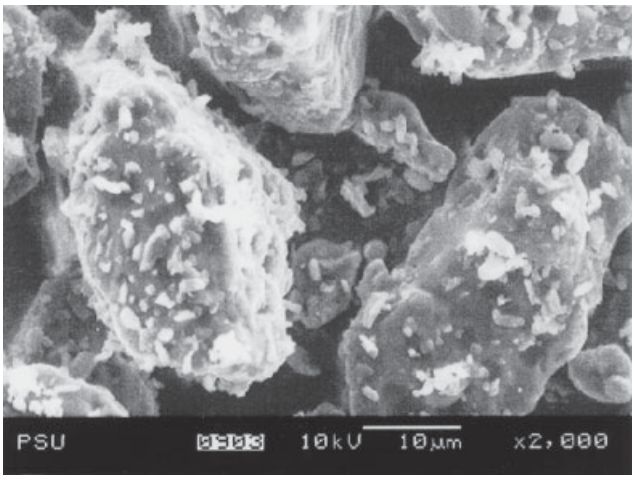

Fig. 1: Small drug particles adhere on large lactose carrier of dry powder formulation containing salbutamol sulphate and lactose at a ratio of 1 : 32.5

2.2. Efficiency of dry powder delivery

The device employed in this study had an internal specific resistance of 0.05 l/min/(mbar), which is classified as intermediate flow resistance [6]. Three holes were employed to generate a turbulence flow in the device. It was found that those two formulations (one containing drug to lactose 1 : 65 and another 1 : 32.5) delivered 50% nominal dose in the upper stage and 40% in the lower stage of the twin stage impinger (TSI). This division of upper and lower stage represented the upper and lower airways, respectively. This device delivered reasonably well as compared to other commercially available devices [7]. The efficacy of the product depends on the interaction of many factors, including the performance of the device, formulation and co-ordination and the inspiratory function of the patient.

2.3. Validation of HPLC-UV and spectrofluorometry analytical methods

2.3.1. HPLC-UV

The intraday and interday accuracy and precision of analytical methods at three levels of concentration are presented in Table 1. The accuracy varied between 99.5 to 101.5% which was acceptable for formulation assays. The intraday precision was less than 1% CV which was a generally acceptable level [8]. The interday variation was less than 2%. The limit of detection was 100 ng/ml. The linearity was obtained over a concentration range of 0.5–2.5 µg/ml ($y = 0.577x + 4.9 \cdot 10^{-4}$, $r^2 = 0.9999$ where x is a concentration in µg/ml and y is a peak area ratio of salbutamol sulphate to ethyl paraben at each concentration).

2.3.2. Spectrofluorometry

A suitable excitation wavelength is 218 nm with the corresponding emission maximum at 309 nm as shown in Fig. 2A. These measurements indicate that the maximum value of salbutamol concentration at which linearity can be obtained is about 2 µg/ml. A calibration graph was constructed by plotting relative fluorescence intensities versus concentration of salbutamol. When lactose was spiked in a fixed concentration of 20 µg/ml the concentration of the drug varied. The fluorescence intensity varied due to drug concentration only. The results showed that lactose did not interfere with the excitation and emission spectra of salbutamol sulphate. The excitation and emission spectra of salbutamol containing lactose were not different from those obtained from pure standard as shown in Fig. 2B. There was no difference in fluorescent intensity between those solutions containing drugs with or without lactose at all concentrations studied as shown in Table 2. As observed, lactose did not generate a measurable signal at concentrations of less than 50 µg/ml. The amount of lactose in each capsule was either 13.5 or 27 mg, therefore

Table 1: Accuracy and precision of salbutamol found in three different concentration analyzed by HPLC-UV (mean ± sd, n = 10)

Concentration (µg/ml)	Intraday run		Interday run	
	Accuracy	Precision (% CV)	Accuracy	Precision (% CV)
0.5	99.54 ± 0.72	0.72	100.27 ± 1.50	1.50
1.5	100.73 ± 0.55	0.55	100.17 ± 1.50	1.29
2.0	101.35 ± 0.45	0.56	101.35 ± 0.45	0.82

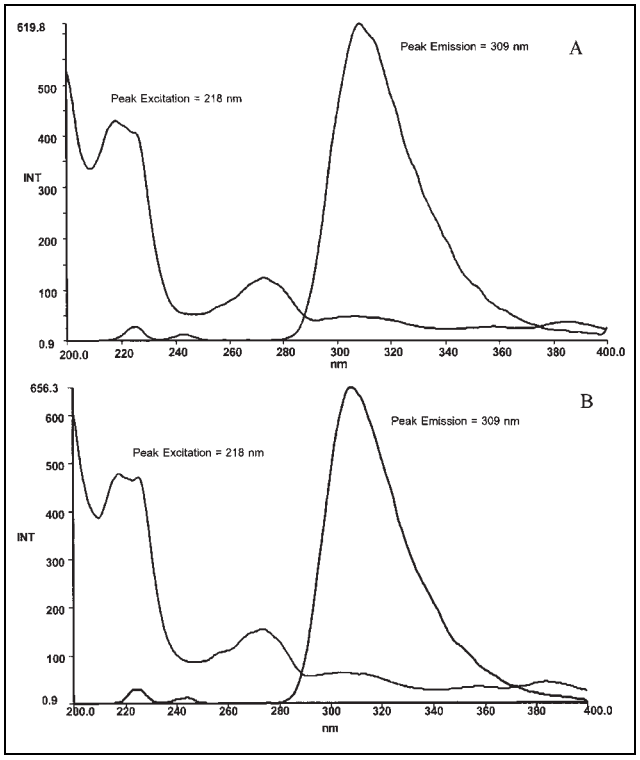

Fig. 2: The excitation and emission spectrum of (A) salbutamol sulphate at a concentration of 1.0 μg/ml in water and (B) salbutamol sulphate at 1.0 μg/ml spiking with lactose at a concentration of 20 μg/ml

the resultant concentration of lactose in all sample solutions was approximately 270 μg/ml. The result showed that the concentration of lactose did not interfere with the determination of salbutamol in the analysis of salbutamol deposition. Moreover, the difference in fluorescence intensity of salbutamol sulphate at different amounts of spiked lactose was found to be statistically insignificant ($p > 0.05$). The linearity of different drug concentrations was obtained from this method ($y = 437.74x + 16.37$; x = concentration in μg/ml and y = intensity). The limit of detection was 0.1 μg/ml.

2.4. Drug delivered from glass delivery devices onto each stage of the ACI analysis by HPLC-UV and fluorometry

Fig. 3 depicts the particle size distribution of a single dose delivery from a salbutamol dry powder inhaler entering the cascade impactor as measured by HPLC-UV and fluorometry. Both formulations were aerosolized at flow rates of 30 and 60 l/min. The total amount of salbutamol collected in the cascade impactor was 265.4 ± 6.53 and 266.25 ± 7.80 μg from those obtained from HPLC-UV and fluorometry, respectively. While the amount of respirable salbutamol (FPF) (sum of stage 2 to filter or

Table 2: Intensity of salbutamol sulphate solution containing with or without lactose

Salbutamol sulphate solution (μg/ml)	Intensity of pure drug solution	Intensity of drug solution containing lactose 20 μg/ml
0.5	238.098	240.490
1.0	453.367	450.008
1.5	665.967	663.221
2.0	896.809	868.481

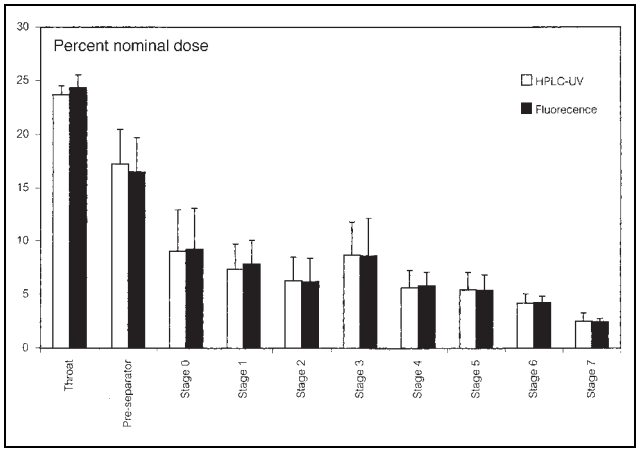

Fig. 3: Size distribution of salbutamol salphate on each stage of the ACI as analyzed by HPLC-UV and spectrofluorometry as aerosolized from a formulation containing drug and carrier ratio of 1 : 32.5 at a flow rate of 60.0 l/min. (mean ± sd, n = 5)

with an MMAD <5.7 μm) was 130.76 ± 5.64 and 130.64 ± 4.88 μg as a similar comparison of the total amount. The amount of drug delivery was formulation and flow rate dependent. Drug particles were delivered to stage 4 when the formulation was aerosolized at 30 l/min (data not shown) but with the same formulation when the flow rate was 60 l/min, drug traveled further to stage 7 (Fig. 3). The y axis was obtained by transformation of the percent cumulative oversize on various stage of the ACI to a probability scale (Z value) and x axis was on logarithm of size (Fig. 4). The MMAD corresponds to Z value of 0 and the GSD was obtained by the size at Z value of 1 divided by the size obtained at Z value of zero. The MMAD and GSD obtained from all conditions in this study are summarized in Table 3 as this was used to compare in the differences of both techniques. The results obtained from the ACI employing those two different analytical methods (HPLC-UV and spectrofluorometry) gave no significant difference in MMAD and GSD ($P > 0.05$).

3. Experimental

3.1. Materials

Salbutamol sulphate was obtained from Alchem International (Berks, UK). Spray dried lactose was purchased from DMV (The Netherlands). Glacial acetic acid was from J. T. Baker (Philadelphia, USA). Heptane sulfonic acid was received from Sigma International (St. Louise, USA). All organic solvents were purchased from J. T. Baker (Philadelphia, USA).

3.2. Dry powder formulations

The sieved lactose (10–40 μm; measured by laser diffraction) was used as a carrier. Micronised salbutamol was mixed with carrier lactose for 30 min in a cylindrical glass tube hold by a Turbular mixer (Basel, Switzerland). Two formulations of dry powders at a drug-carrier ratio of 1 : 65 and

Table 3: The MMAD and GSD as obtained from ACI analyzed either with HPLC-UV or spectrofluorometry from two dry powder aerosols containing drug to carrier ratio of 1 : 32.5 and 1 : 65 aerosolized at 30 and 60 l/min

Flow rate (l/min)	Drug carrier ratio	MMAD HPLC-UV	GSD	MMAD Fluorescence	GSD
30	1 : 32.5	4.0619	1.4999	4.1455	1.4802
	1 : 65	4.1864	1.4676	4.1888	1.4676
60	1 : 32.5	2.8416	1.8034	2.9245	1.8381
	1 : 65	2.8427	1.8048	2.8888	1.7724

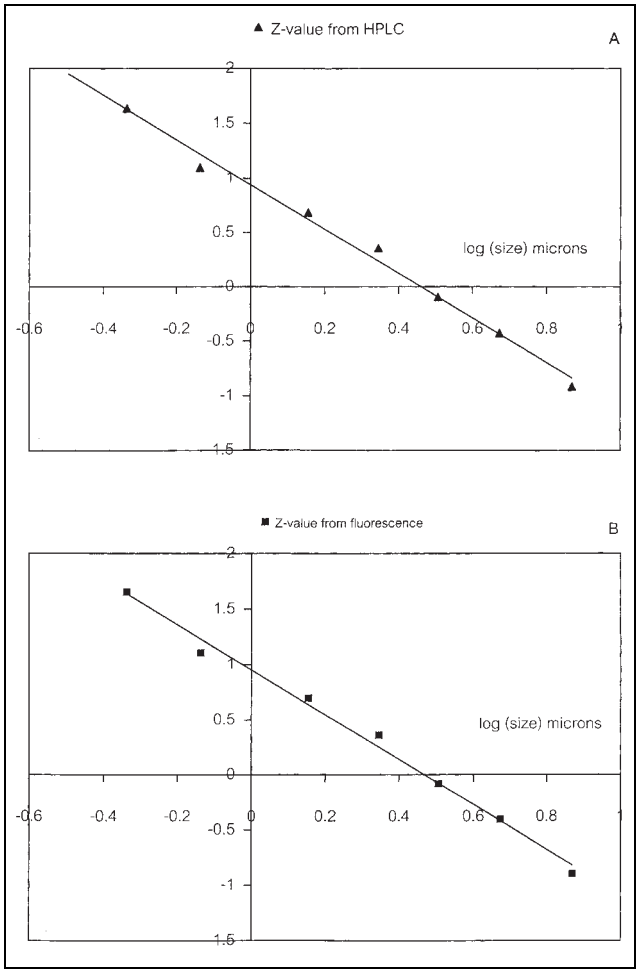

Fig. 4: Relationship between Z value and cut off aerodynamic diameter (log scale) on each stage of the ACI obtained from (A) HPLC-UV and (B) Spectrofluorometry analysis as aerosolized a formulation containing drug and carrier ratio 1 : 32.5 at a flow rate of 60.0 l/min

1 : 32.5 by weight were formulated. The content uniformity of the formulations was confirmed prior to further study by analyzing the amount of salbutamol found in the blend as compared to theoretical value. The drug was analyzed by the HPLC-UV method described in following section. Moreover, the orientation of the drug and carrier was observed by SEM equipped with energy dispersive X-ray [9]. Then, 26.4 and 13.4 mg of the blends, equivalent to 400 μg drug, were weighed into each capsule.

3.3. Dry powder delivery system

The dry powder inhaler device made in-house was employed to evaluate dry powder formulations (Fig. 5). The device has a 29 Quickfit socket fitted to a sample port. The sample port allows the powder to be introduced from the capsule into the device prior to aerosolization. A plastic grid (74 μm) was fitted to deaggregate the large particle sizes. The bleed holes reduce the resistance of the device and generate turbulence inside the device. The device resistance is measured according to a method used elsewhere [6]. A glass inhaler device was designed and made at Prince of Songkla University. The dry powder formulation was charged into the ACI that is official in the EP 1997 [3]. Dry powder formulation was loaded into the cone shape funnel of the device before drying air was drawn through the mouthpiece of the device to the ACI at a flow rate of 30 and 60 l/min.

3.4. Validation of HPLC-UV

Salbutamol was analyzed by HPLC (Waters, CA, USA) using a UV detector set at a wavelength of 276 nm. The stationary phase was μ-bondapak C18 (30 cm × 3.9 mm). The mobile phase was 0.06% heptane sulfonic acid (adjust to pH 4.5 by glacial acetic acid) and methanol at ratio of 60 : 40 by volume. Then the mobile phase was filtered through a Nylon membrane (Whatman, USA) before use. The flow rate was 1 ml/min. Stock solution of salbutamol sulphate was prepared in distilled water at a concentration of 50 μg/ml. Afterwards, a solution of salbutamol sulphate was diluted to 5 different concentrations; 0.5, 1.0, 1.5, 2.0 and 2.5 μg/ml with distilled water. The internal standard was ethyl paraben at a concentration of 0.2 μg/ml. The injection volume was 20 μl. The standard curve was constructed with those 5 concentrations. The interday and intraday accuracy and precision was monitored at a concentration of 0.5, 1.5 and 2.0 μg/ml.

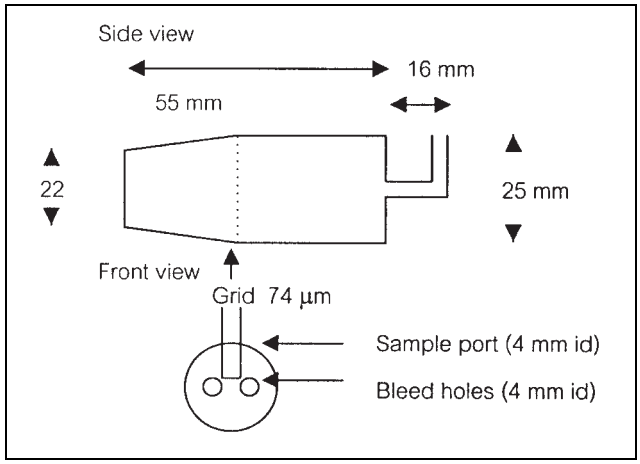

Fig. 5: Diagram of glass inhaler device showing dimensions which fitted the glass throat of a twin stage impinger (BP 1998).

3.5. Validation of spectrofluorometry

All fluorescence measurements were done on a Perkin Elmer equipped with a 150 W xenon lamp. These experiments were done on a spectrofluorometer using a 1 cm quartz cell. The stock solution was prepared once a week and stored in a refrigerator before measuring the fluorescence. Solutions for the calibration curve were prepared by convenient dilution of stock solution with water in order to obtain a concentration range of 0–20 μg/ml. Fluorescence intensity was measured immediately at 310 nm (excited at 290 nm). Linear regression was used to determine slopes, intercept and correlation coefficient over 5 concentrations for 5 consecutive days. This was done 5 times a day at each concentration. Salbutamol sulphate solutions containing 5 different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 μg/ml) as described above were analyzed by spectrofluorometry (Perkin Elmer, USA). The intensity of fluorescence was measured to prepare the standard curve. The absorption and emission spectra were recorded at 5 mm slit width. Linearity was calculated over a range of concentrations. The effect of carrier on fluorescence intensity was tested by two procedures; first, the concentration of lactose was maintained at 20 μg/ml and the concentration of salbutamol was varied similarly to that of a standard curve and secondly, the concentration of lactose was varied as following: 10, 20, 30, 40 and 50 μg/ml while a concentration of salbutamol sulphate was at 1 μg/ml. The fluorescence intensity was measured as described before. This was to verify the effect of lactose concentration on the fluorescence intensity of salbutamol. Paired t-test was used to compare intensity differences. The percent relative standard deviation (%RSD) of measuring intensity was calculated.

3.6. Drug delivery from glass delivery devices

The inhaler device was loaded with dry powder aerosols for either 13.4 and 26.4 mg of formulations containing salbutamol sulphate 400 μg. The airflow was drawn through the device at a flow rate of 30 and 60 l/min for 20 and 10 s, respectively. This was delivered into the ACI and the experiment was carried out 5 times. For each delivery, the drug deposition on each plate and stage of the ACI was analyzed by either HPLC-UV or spectrofluorometry. In the case of HPLC, the sample on each stage was washed with mobile phase containing internal standard, in the case of spectrofluorometry, only distilled water filtered with 0.45 μm was employed. Then the eluent was adjusted to the appropriate volume with the respective solvent. Data interpretation was done in two ways. Particle size diameters were generated by plotting effective cut-off as the ordinate and a cumulative percentage undersize as the abscissa on log probability scale. Further, the best straight line was drawn through data points. The MMAD is represented by the value of effective cut-off diameter at cumulative percentage of 50%, GSD equals the quotient of the MMAD divided by the value of ECD at cumulative percentage of 84% ($z = 1$). Replicate results of 5 experiments gave a mean of MMAD and GSD. In this work, values of MMAD and GSD from HPLC-UV were compared with those obtained from spectrofluorometry. Size distribution and percentage deposition on each stage was plotted on log-probability scale (log-Z value). Drug deposition on each stage was calculated for MMAD and GSD as described elsewhere [10]. All experiments were carried out at 25.1 ± 1.38 °C with a relative humidity of 58.81 ± 5.40%. The data were analyzed for statistical significance using a paired t-test.

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